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Enterotoxigenic *Escherichia coli* (ETEC) is capable of invading epithelial cell lines derived from the human colon and ileocecum. Two separate loci (*tia* and *tib*) that direct the non-invasive *E. coli* strain HB101 to adhere to and invade intestinal epithelial cells have previously been cosmid cloned from ETEC strain H10407. SDS-PAGE analysis of cellular fractions from *tib*-positive HB101 shows that the *tib* locus directs the synthesis of a 104 kDa outer membrane protein (the TibA protein). The *tib* locus was subcloned to a maximum of 6.7 kb and mutagenized with transposon Tn5. Production of TibA was directly correlated with the capacity of the subclones and Tn5 mutants to invade and adhere to epithelial cells, suggesting that TibA was required for these phenotypes. The position and direction of transcription of the *tibA* gene was identified by complementation and in vivo T7 RNA polymerase/promoter induction experiments. The role of the *tib* locus in epithelial cell invasion was confirmed by the construction of chromosomal deletion derivatives in H10407. These deletion mutants invaded epithelial cells at about 15% of the parental level and were fully complemented by plasmids bearing the *tib* locus.

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Epithelial Cell Invasion and Adherence Directed by the Enterotoxigenic *Escherichia coli tib* Locus Is Associated with a 104-Kilodalton Outer Membrane Protein

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Enterotoxigenic *Escherichia coli* (ETEC) is capable of invading epithelial cell lines derived from the human colon and ileocecum. Two separate loci (*tia* and *tib*) that direct noninvasive *E. coli* HB101 to adhere to and invade intestinal epithelial cells have previously been cosmid cloned from ETEC H10407. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of cellular fractions from *tib*-positive HB101 shows that the *tib* locus directs the synthesis of a 104-kDa outer membrane protein (the TibA protein). The *tib* locus was subcloned to a maximum of 6.7 kb and mutagenized with transposon Tn*5*. Production of TibA was directly correlated with the capacity of the subclones and Tn*5* mutants to invade and adhere to epithelial cells, suggesting that TibA was required for these phenotypes. The position and direction of transcription of the *tibA* gene were identified by complementation and in vivo T7 RNA polymerase-promoter induction experiments. The role of the *tib* locus in epithelial cell invasion was confirmed by the construction of chromosomal deletion derivatives in H10407. These deletion mutants invaded epithelial cells at about 15% of the parental level and were fully complemented by plasmids bearing the *tib* locus. The size and function of the TibA protein are similar to those of invasin from *Yersinia pseudotuberculosis* (103 kDa). However, a *tib* probe did not hybridize with the gene encoding invasin. Hybridization analyses of genomic DNA from a wide variety of pathogenic and nonpathogenic bacteria, including *Salmonella*, *Shigella*, *Yersinia*, and *Escherichia* species, indicate that the *tib* locus is unique to specific ETEC strains.

Enterotoxigenic *Escherichia coli* (ETEC) infection results in over 600 million cases of diarrhea worldwide annually (16). ETEC disease is initiated by consumption of contaminated food or water. Bacteria transit to and colonize the upper small bowel and produce heat-stable and/or heat-labile enterotoxins (25). Organisms attach to the intestinal mucosa via fimbrial colonization factor antigens (CFAs). Several different CFAs have been described in ETEC. Proliferation of an enterotoxin or enterotoxins results in the watery diarrhea associated with ETEC infection. However, the production of enterotoxins may not be exclusively required for diarrhea (26, 28, 29, 33, 40).

Penetration of the intestinal mucosa is not thought to be a pathogenic trait of ETEC. However, it has been found that these organisms are capable of invading specific epithelial cell lines and are most invasive for epithelial cells derived from the human ileocecum and colon (10). Two separate, chromosomally encoded invasion determinants (the *tia* and *tib* loci) were cloned from the classical ETEC strain H10407 and were expressed in *E. coli* HB101. These loci could direct HB101 to adhere to and invade HCT 8 cells (human ileocecal epithelium) with an invasion efficiency of 100 to 150% of that of the parental ETEC strain. H10407 and HB101 containing either the *tia* or *tib* locus could transit through polarized HCT 8 monolayers, suggesting that ETEC may be crossing the gut epithelium in vivo and that epithelial cell penetration may have a previously unrecognized role in enterotoxigenic disease (10).

This report presents a further characterization of the ETEC *tib* locus. The *tib* locus directs the synthesis of a 104-kDa outer membrane protein (OMP). Subcloning and transposon mutagenesis experiments show that production of this protein is directly correlated with the adherence and invasion phenotypes. Deletion of wild-type *tib* sequences from ETEC H10407 decreases epithelial cell invasion to about 15% of the parental level. Hybridization of genomic DNA from several enteric pathogens, including *Salmonella*, *Shigella*, *Yersinia*, and *Escherichia* species, to a *tib* probe indicates that this locus is found only in specific ETEC strains.

MATERIALS AND METHODS

Bacterial strains, plasmids, tissue culture cells, and culture conditions. ETEC H10407 (11 [serotype O78:H11; CFA/I]) was the parent strain in cloning experiments. H10407S, a spontaneous Sm^r derivative of H10407, was isolated by plating 10^{10} H10407 cells on agar media containing streptomycin. *E. coli* HB101 (1 [*hsdS20 recA13 rpsL20*]) was used as a noninvasive recipient strain for recombinant plasmids. Organisms were grown in Luria broth (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl [pH 7.6] per liter) at 37°C and 200 rpm, unless indicated otherwise. Where indicated, antibiotics were added to growth media to the following final concentrations: ampicillin, 100 μg/ml; kanamycin, 20 μg/ml; and streptomycin, 100 μg/ml. The plasmids used in this study are listed in Table 1.

HCT 8 (38 [ATCC CCL 244]) epithelial cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum, 1 mM sodium pyruvate, and 2 mM L-glutamine. HCT 116 (ATCC CCL 247), HuTu 80 (12 [ATCC HTB 40]), and HEp-2 (ATCC CCL 23) cell lines were maintained in minimal essential medium containing 10% fetal bovine serum, 1 mM sodium pyruvate, and 2 mM L-glutamine. Epithelial cells were grown at 37°C in a 6% CO_2 atmosphere, and gentamicin was added to 100 μg/ml where indicated.

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TABLE 1. Plasmids used in this study

Plasmid	Relevant description	Reference
pACYC184	Cloning vector; p15A replication origin	4
pCVD437	EPEC *eae* gene	21
pCVD442	Positive-selection suicide vector	7
pET102	ETEC *tia* invasion locus from H10407	10
pET103	ETEC *tib* invasion locus from H10407	10
pHC79	Cosmid cloning vector	15
pHG165	pBR322 copy number derivative of pUC8; ColE1 replication origin	35
pRI203	*Y. pseudotuberculosis inv* gene	17
pT7-5	T7 RNA polymerase promoter expression vector	36
pT7-6	T7 RNA polymerase promoter expression vector	36
pTY19	*S. typhi* invasion loci *invA*, *-B*, *-C*, and *-D*	9

Invasion and adherence assays. Bacterial invasion of epithelial cells was measured as protection from the bactericidal antibiotic gentamicin (22). Invasion and adherence assays were performed as previously described (10). The results of invasion and adherence assays are variable on a daily basis; therefore, the datum points presented in the figures and tables are average values (± range) from triplicate wells of a single experiment and are representative of values obtained in replicate experiments.

Membrane fractionation. Cellular fractions were isolated as described by Schnaitman (30). Luria broth cultures (500 ml each) were grown with shaking at 37°C to late log phase, harvested by centrifugation, and then lysed by two passages through a French press. Cytosolic fractions were separated from membrane fractions by ultracentrifugation at 100,000 × *g* for 1 h. Inner and outer membranes were isolated by sucrose density gradient centrifugation (30). The extent of cross-contamination between cellular fractions was not determined. In sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses, the major OMPs served as markers for the outer membrane fractions and for contamination of other fractions by the outer membrane. Because the extent of cross-contamination was not measured, the cellular location of proteins cannot exclusively be associated with a single fraction.

SDS-PAGE analysis of proteins. Electrophoresis of whole-cell extracts or membrane fractions was performed under denaturing conditions by the method of Laemmli (23). Samples were prepared for electrophoresis by heating in treatment buffer at 95°C for 10 min. Gels were run for 16 to 18 h at 40 V at room temperature. Gels were stained for protein with Coomassie blue. The protein concentration of membrane fractions was determined by the Bradford method (2). The following quantities of protein were loaded per well for SDS-PAGE analysis: outer membrane, 20 μg; inner membrane, 60 μg; and cytosol, 120 μg. For the analysis of whole-cell extracts, 5-ml Luria broth cultures were grown to late log phase by shaking at 37°C and then were harvested by centrifugation and lysed in 200 μl of treatment buffer. Twenty-microliter aliquots were loaded on SDS-PAGE gels.

For immunoblotting, freshly prepared cellular fractions were treated for electrophoresis as described above. However, samples containing invasin were incubated in treatment buffer at 37°C for 10 min. After electrophoresis, separated proteins were electrotransferred to nitrocellulose filters at 50 V for 90 min in standard buffer (39). The filters were blocked with Tris-buffered saline solution (10 mM Tris hydrochloride [pH 7.5], 150 mM NaCl) containing 2% (wt/vol) casein. Monoclonal antibodies against invasin (24) were diluted 1:40 in casein blocker and incubated with nitrocellulose filters for 2 h at room temperature. After washing of the filters, bound antibody was detected with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (Kirkegaard and Perry, Gaithersburg, Md.).

Complementation analysis. HB101 was transformed with the indicated plasmids, and whole-cell extracts were prepared as described above. Plasmid pHG165 was the vector for plasmids pET113, pET139, and pET140. Plasmid pACYC184 was the vector for plasmid pET146. Since the *lac* promoter of pHG165 was derepressed in HB101, the complementation experiments were performed in the absence of isopropyl-β-D-thiogalactopyranoside (IPTG). The same results were obtained if 2 mM IPTG was added to the growth medium.

Radiolabeling of plasmid-encoded proteins. Specific in vivo radiolabeling of plasmid-encoded proteins by a T7 RNA polymerase-promoter expression system (36, 37) was performed with *E. coli* K165 (5) with M9 minimal salts medium supplemented with 0.5 mg of thiamine per ml and 2% methionine assay medium (Difco Laboratories, Detroit, Mich.). Plasmid pT7-5 was the vector for plasmids pET172 and pET175. Plasmid pT7-6 was the vector for plasmids pET156 and pET176. Samples (40 μl) from each labeling were heated at 95°C for 10 min and then electrophoresed under denaturing conditions through SDS–7.5% polyacrylamide gels. Labeled proteins were detected by autoradiography of dried gels.

Transposon Tn*5* mutagenesis. *E. coli* EM3001 (8 [*hsdR$_B$11 recA56*]) containing pET109 was infected with the Tn*5* vector λNK467 (6). Plasmids from Km^r colonies were purified and used to transform HB101 to Ap^r and Km^r. Transformants were screened for the ability to invade HCT 8 cells and for the production of the 104-kDa OMP. The points of Tn*5* insertion were determined by digestion of the mutagenized plasmid with *Eco*RI, *Bam*HI, *Hin*dIII, and *Sal*I.

Construction of Δ*tib* derivatives. A *tib* deletion was constructed by *Ava*I digestion and religation of pET113 to generate pET165 (see Fig. 7). The insert of pET165 is flanked by *Hpa*I sites present in the vector pHG165. Plasmid pET165 was digested with *Hpa*I, and a 3,235-bp fragment containing the *tib* deletion was purified by gel electrophoresis and elution and then ligated into the *Sma*I site of the positive-selection suicide vector pCVD442 to generate pET167. Plasmid pET167 was conjugally transferred to H10407S from *E. coli* SM10λ*pir* (31 [*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km]) by plate mating, with selection for Ap^r and Sm^r. Purified transconjugants were grown to late log phase, diluted, and used to inoculate plates containing tryptone (10 g/liter), yeast extract (5 g/liter), and sucrose (50 g/liter). These sucrose selection plates were incubated at 30°C. Purified sucrose-insensitive colonies were screened for the loss of Ap^r, and Ap^s strains were examined for the loss of *tib* sequences by hybridization analysis with probes I and II (Fig. 7).

Hybridization analysis. Three *tib* probes were generated from pET109 by restriction endonuclease digestion. Probe I was a 5.8-kb *Bam*HI fragment, probe II was a 2.1-kb *Bam*HI-*Hin*dIII fragment, and probe III was a 1.9-kb *Sal*I-*Hpa*I fragment (Fig. 7). Probe DNAs were purified by preparative electrophoresis and elution from 0.8% agarose gels and then were random-primer labeled with digoxigenin-dUTP (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) or [α-^{32}P]dCTP. Genomic DNAs were prepared by hexadecyltrimethyl ammonium bromide precipitation of bacterial lysates (41). For genomic hybridizations, approximately 1 μg of DNA was digested with *Bam*HI and then electrophoresed through 0.6% agarose gels. DNA was transferred to Nytran (Schleicher

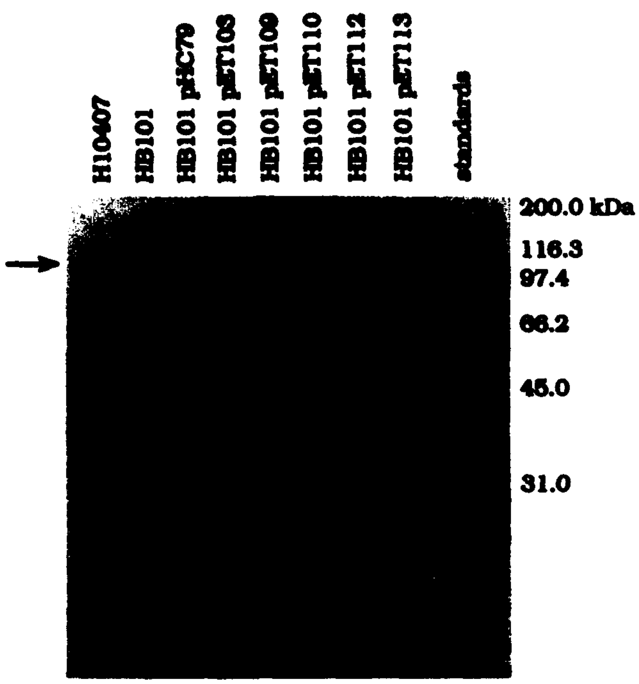

FIG. 1. TibA production by HB101 containing the *tib*-positive plasmid pET103 or various subclones. Plasmids are shown in Fig. 3. Whole-cell extracts were separated by electrophoresis through an SDS–13% polyacrylamide gel and then stained with Coomassie blue. Molecular mass standards are indicated on the right of the figure. The arrow on the left indicates the migration of TibA (the 104-kDa protein).

and Schuell, Keene, N.H.) by the method of Southern (34). Hybridization and detection of digoxigenin-labeled probes were performed according to the manufacturer's protocols (Boehringer). Hybridization and stringency washes were performed at 40°C. Stringency washes were done in 0.1% SDS–0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate).

RESULTS

The *tib* locus directs the synthesis of a 104-kDa outer membrane protein. Plasmid pET103 was isolated from an H10407 library by screening for cosmids that could direct HB101 invasion of HCT 8 (human ileocecum epithelial) cells. The invasion system encoded by pET103 is referred to as the *tib* locus (10). SDS-PAGE analysis of whole-cell extracts revealed a unique protein that was synthesized by HB101 carrying pET103 (Fig. 1). This protein was designated TibA. TibA was not seen in whole-cell extracts of H10407, the parent ETEC strain. Figure 1 is an SDS-PAGE gel (13% polyacrylamide) that indicates TibA has a molecular mass of nearly 116 kDa. However, after multiple SDS-PAGE analyses on lower-percentage gels, the average molecular mass of TibA was estimated to be 104 kDa.

To identify the cellular location of TibA, inner membranes, outer membranes, and cytosolic fractions were isolated from HB101 carrying the cosmid cloning vector pHC79, HB101 carrying *tib* plasmid pET103 or pET113, and H10407. SDS-PAGE analysis of these fractions indicated that TibA was found predominantly in the outer membrane of HB101 carrying pET103 or pET113 (Fig. 2). TibA was the only protein in

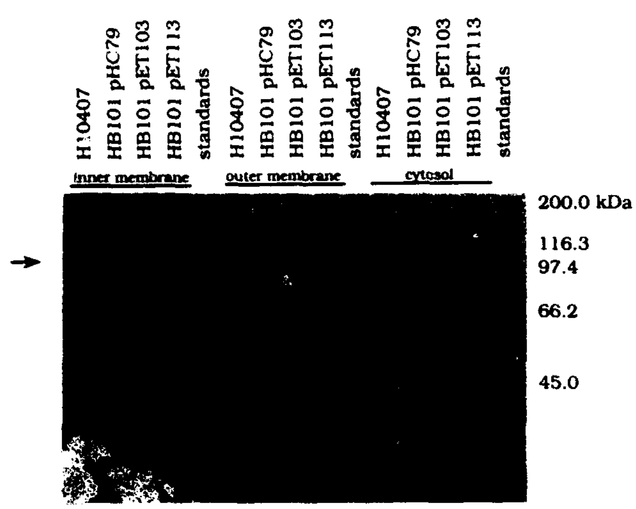

FIG. 2. Coomassie blue-stained SDS-PAGE gel (7.5% polyacrylamide) of cellular fractions. Molecular mass standards are indicated on the right of the figure. The arrow on the left indicates the migration of TibA.

extracts of *tib*-positive HB101 not observed in extracts of HB101(pHC79). There appeared to be little or none of this protein in the outer membrane of H10407. Outer membranes from H10407 contained a protein that was similar in size but slightly smaller than TibA.

Subcloning and mutagenesis of the *tib* locus. Subcloning of pET103 identified 8.1 kb of sequence associated with the invasive phenotype (Fig. 3). The ability of subclones to adhere to and invade HCT 8 cells was directly correlated with the production of the TibA protein. All subclones that produced TibA remained invasive and adherent, whereas all subclones that did not produce TibA were not invasive or adherent (Fig. 1 and Table 2). As the *tib* insert size was progressively reduced

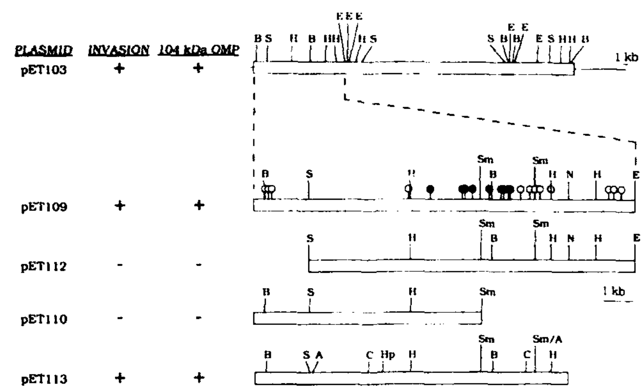

FIG. 3. Subcloning and mutagenesis of the *tib* locus. Cosmid pET103 carries the *tib* locus and was the parent plasmid for subcloning. The ability of the subclones to direct invasion and production of TibA (104-kDa OMP) is indicated to the left of the restriction maps. Points of Tn*5* insertion are shown on the map of pET109. Solid circles indicate the positions of insertions that eliminate invasion and production of TibA. Open circles indicate the positions of insertions that have no effect on invasion or TibA production. Restriction enzymes: A, *Ava*I; B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; N, *Nru*I; S, *Sal*I; Sm, *Sma*I. There are additional unmapped *Ava*I sites between the two sites shown.

TABLE 2. Invasion of and adherence to HCT 8 epithelial cells by *tib* subclones

Strain[a]	% Invasion[b]	% Relative invasion[c]	% Adherence[d]	% Relative adherence[e]
HB101	0.01 ± 0.01	0.6	0.28 ± 0.05	9.1
pET103	1.64 ± 0.05	100	3.09 ± 0.13	100
pET109	1.76 ± 0.18	107.3	5.54 ± 0.14	179.3
pET110	0.05 ± 0.05	3.0	0.41 ± 0.15	13.3
pET112	0.06 ± 0.02	3.7	0.37 ± 0.02	12.0
pET113	1.87 ± 0.12	114.0	8.13 ± 0.80	263.1

[a] All strains listed are HB101, with or without the indicated plasmid.
[b] Percentage of inoculum resisting treatment by gentamicin.
[c] Invasion relative to HB101(pET103), representing 100%.
[d] Percentage of inoculum remaining on washed monolayers at 3 h (minus percentage of inoculum resisting gentamicin treatment).
[e] Adherence relative to HB101(pET103), representing 100%.

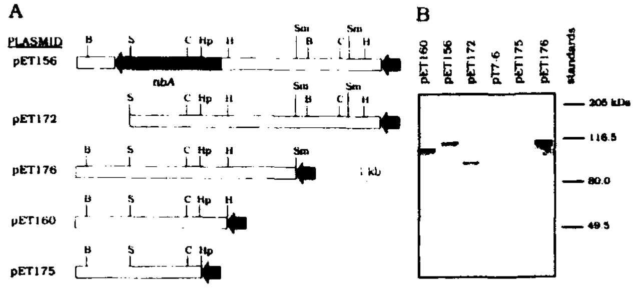

FIG. 4. T7 RNA polymerase-promoter in vivo radiolabeling of plasmid-encoded proteins. (A) Restriction maps of *tib* locus subclones. Plasmids pET156 and pET113 contain the same insert DNA. The direction of transcription of the T7 RNA polymerase promoter is indicated by solid arrows. The position of the *tibA* gene is based upon the size of the truncated protein produced by pET172. Restriction enzymes are as described in the legend to Fig. 3. (B) Autoradiograph of an SDS-PAGE gel (7.5% polyacrylamide) showing peptides synthesized by strains bearing the plasmids in panel A. Molecular mass standards are indicated on the right of the figure.

from 34.4 kb (in pET103) to 10.2 kb (in pET109) to 8.4 kb (in pET113), the percentage of invasion of active subclones increased slightly compared with that of HB101(pET103). However, the percentage of adherence of active subclones increased to a greater extent than did the percentage of invasion (Table 2). This increase in adherence did not appear to be associated with an increase in TibA production as observed in outer membrane preparations (Fig. 2). The meaning of this increase in adherence is unknown.

Tn*5* insertion mutants of pET109 were isolated and then screened for the ability to direct epithelial cell invasion and to produce TibA (Fig. 3). As found with the subclones, there was a direct correlation between the production of TibA and the ability of the transposon mutants to adhere to and invade epithelial cells. Every insertion mutant that produced TibA remained invasive and adherent, whereas all mutants that did not produce TibA were not invasive or adherent. All of the invasion-negative insertions were found in a region of pET109 that had been identified as necessary for invasion by subcloning experiments.

On the basis of the position of flanking insertions that did not affect invasion, the size of the transposon-identified invasion-associated region was about 3.5 kb. This amount of DNA corresponds well to the molecular mass of TibA, which would require about 2.9 kb of coding capacity, suggesting that this region may contain the *tibA* gene. However, during the Tn*5* mutagenesis of pET109, the *tib* locus was not saturated with insertions and there remained a segment of the locus large enough to encode the TibA protein. Subcloning experiments indicated that this segment of the *tib* locus also was required for epithelial cell entry (Fig. 3).

Localizing the *tibA* gene. To further clarify the position of the *tibA* gene, *tib* locus subclones were constructed that were under the control of an exogenous T7 promoter. In vivo protein synthesis directed by this promoter was analyzed (Fig. 4). Plasmid pET156 contained the same insert carried by plasmid pET113 and directed the synthesis of a single high-molecular-mass peptide of about 104 kDa. When the *tib* locus was placed behind the T7 promoter in an orientation opposite to that shown for pET156, there were no specific peptides synthesized, indicating the direction of *tibA* transcription. Plasmid pET172 carried a deletion of 3′ *tib* sequences and produced a 93.5-kDa truncated version of TibA. On the basis of the size of this truncated protein, the position of the *tibA* gene was estimated (Fig. 4). According to this estimate, pET160 should contain the entire *tibA* gene but produced a 100-kDa form of the TibA protein. Removal of 5′ *tibA* sequences resulted in the loss of TibA synthesis (pET175 [Fig. 4]). If sequences upstream of the *tibA* gene were included, a full-length TibA protein was observed (pET176 [Fig. 4]). Although pET176 synthesized a full-length TibA protein, the same insert could not direct invasion or produce TibA in the absence of the T7 promoter (pET110 [Fig. 1 and 3]). To verify that the upstream region was not coding for TibA, the *tib* sequences identified in plasmids pET146 and pET180 (Fig. 5) were placed (in both orientations) under the control of either the T7 or *lac* promoter. These constructs did not produce the 100- or 104-kDa forms of TibA (data not shown). Epithelial cell invasion directed by the T7 promoter plasmids could not be measured, since induction of T7 polymerase was lethal to the strains containing these plasmids.

Complementation experiments. Subcloning and transposon mutagenesis experiments indicated that sequences upstream of the *tibA* gene were required for invasion and TibA synthesis. To examine if this requirement was the result of polarity effects on *tibA* transcription, complementation experiments were performed. Plasmids pET139 and pET140 (Fig. 5) carry the same insert contained by plasmid pET160 (Fig. 4), but with transcription under the control of the *lac* promoter rather than the T7 promoter. When *lac* transcription was opposite to that of *tibA*, no TibA protein was observed in whole-cell extracts (Fig. 5). However, when *lac* transcription was in the same direction as *tibA* transcription, a 100-kDa form of the TibA protein was produced (Fig. 5), a result identical to that obtained when transcription was controlled by the T7 promoter (pET160 [Fig. 4]). This 100-kDa protein was found predominantly in the outer membrane (data not shown). Plasmid pET146 carries the region upstream of *tibA*. While strains carrying pET146 alone did not express TibA, HB101 containing pET146 and either pET139 or pET140 produced a full-length TibA protein (Fig. 5). The same result was observed if pET180 (Fig. 5) was used to complement plasmids carrying the *tibA* insert contained by pET139 and pET140 (data not shown). Invasion of HCT 8 cells directed by these constructs was measured. HB101 carrying pHG165, pACYC184, pET139, pET140, or pET146 did not invade HCT 8 cells (<0.02% invasion). However, HB101 containing pET146 and either pET139 or pET140 invaded HCT 8 cells as efficiently as pET113 (6.80% invasion). These results indicated that the loss of TibA synthesis upon deletion or mutagenesis of the upstream region was not due to polarity effects on *tibA* transcription but that this region was necessary

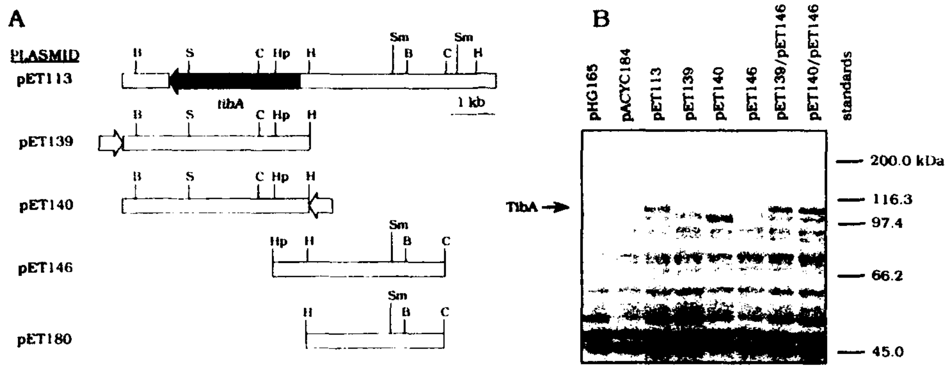

FIG. 5. Complementation of the *tib* locus. (A) Restriction maps of *tib* locus subclones. The direction of transcription of the *lac* promoter is indicated by open arrows. Restriction enzymes are as described in the legend to Fig. 3. (B) Coomassie blue-stained SDS-PAGE gel (7.5% polyacrylamide) of whole-cell extracts of HB101 containing the indicated plasmid or plasmids. Migration of TibA is indicated by the arrow on the left of the figure. Molecular mass standards are indicated on the right of the figure.

for *tibA* transcription. Additionally, these experiments indicated the maximal amount of sequence required for full activity of the *tib* locus was 6.7 kb.

Cell line specificity of the *tib* locus. Previous experiments showed that H10407 was specific for the epithelial cell lines it could invade (10). Additionally, it was found that the *tib* locus could direct HB101 to invade HeLa cells, a cell line not penetrated by H10407 (10). The cell line specificity of the *tib* locus was examined further to determine if the activity of this system reflected the activity of the parent strain. H10407 could invade the HCT 8 and HCT 116 human colonic epithelial cell lines but could not invade HEp-2 cells (human larynx epithelial cells) or HuTu 80 cells (human duodenal epithelial cells). *tib*$^+$ HB101 did not display the cell line specificity of H10407, because it invaded every cell line examined (Fig. 6).

The *tib* locus is required for epithelial cell invasion by H10407. The role of the *tib* locus in epithelial cell invasion was verified by deletion of *tib* sequences from H10407. A deletion that included the majority of the *tibA* gene and the entire upstream invasion-associated region identified by Tn*5* mutagenesis was generated by *Ava*I digestion and religation of pET113 (Fig. 7). The plasmid bearing the *tib* deletion (pET165) did not direct the production of TibA or the invasion of HCT 8 cells by HB101. This deletion mutation (Δ*tib1*) was introduced into H10407S by allelic exchange mutagenesis with the positive-selection suicide vector pCVD422 (7). Two independent deletion derivatives were isolated and designated TIB3 and TIB4. The loss of *tib* sequences was verified by hybridization with probes I and II (Fig. 7). Figure 8 shows the probe I hybridization pattern of *Hin*dIII-digested TIB3 and TIB4 genomic DNA. The Δ*tib1* mutation deletes a *Hin*dIII site found within the *tib* locus (Fig. 7). The absence of this site in TIB3 and TIB4 was seen by the loss of two homologous *Hin*dIII fragments and the appearance of a new junction fragment. Both of the deletion mutants failed to anneal with probe II, indicating that the sequences internal to the deletion had been lost in these strains. Membrane fractions purified from TIB3 and TIB4 appeared to be identical to membranes prepared from the parent strain (data not shown). The ability of TIB3 and TIB4 to adhere to HCT 8 cells was reduced to about one-third of the wild-type level (Fig. 9A). Invasion of HCT 8 cells by these mutants was reduced to about 15% of the parental level (Fig. 9B). Wild-type adherence and invasion activity could be restored to TIB3 and TIB4 by introduction of pET109 (Fig. 9). These results show that the *tib* locus is required for full invasive potential in H10407. The residual

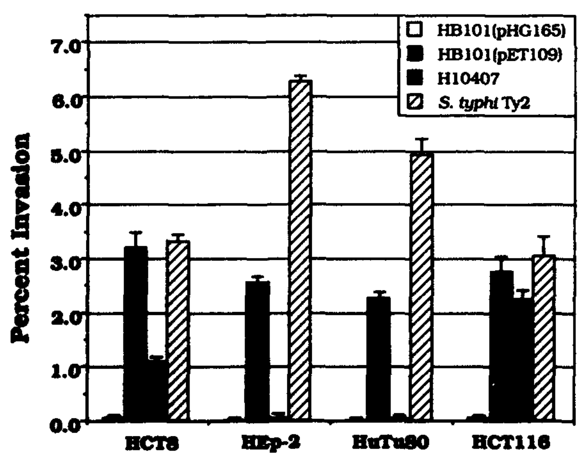

FIG. 6. Epithelial cell line specificity of the *tib* locus. Data are shown as an average of three replicates, with the range shown as a bar above the average.

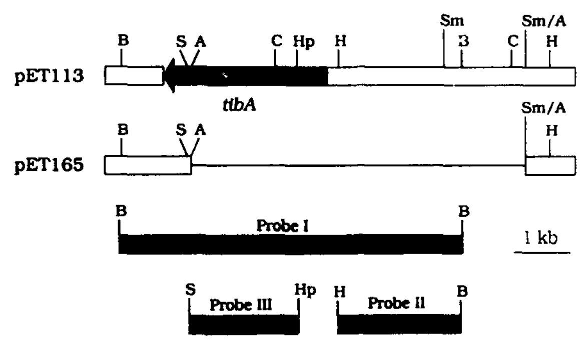

FIG. 7. *tib* locus deletion and probes. Restriction maps of plasmids used in the construction of a *tib* deletion are shown. The thin line in the map of pET165 represents a deletion constructed by *Ava*I digestion and religation of pET113. The sequences comprising probes I, II, and III are indicated below the restriction maps. Restriction enzymes are as described in the legend to Fig. 3.

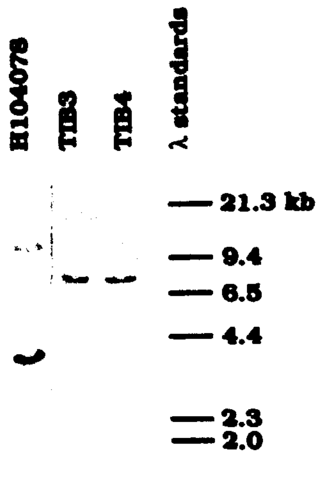

FIG. 8. Hybridization of *tib* deletion derivatives with probe I. Genomic DNAs from the indicated strains were digested with *Hin*dIII, electrophoresed through a 0.6% agarose gel, transferred to nitrocellulose, and then probed with digoxigenin-labeled *tib* probe I (Fig. 7). The positions of λ *Hin*dIII standards are indicated on the right of the gel.

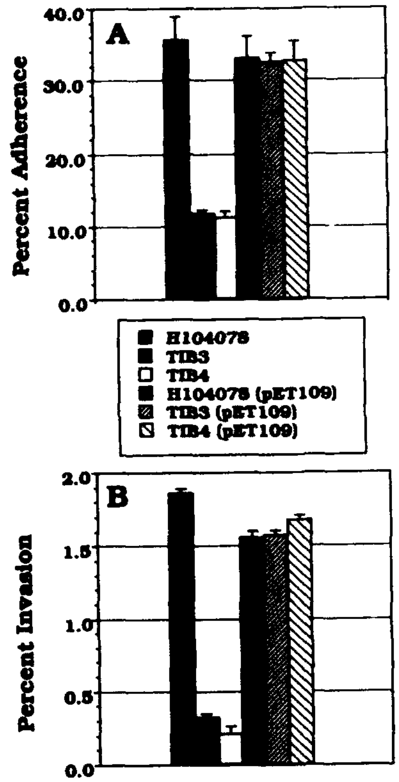

FIG. 9. Activity and complementation of H10407S *tib* deletion derivatives TIB3 and TIB4. (A) Adherence to HCT 8 cells. (B) Invasion of HCT 8 cells. Data are shown as an average of three replicates, with the range shown as a bar above the average.

invasion activity of TIB3 and TIB4 may represent the functioning of the *tia* invasion locus in these strains. These results also show that the *tib* locus plays a major role in epithelial cell adherence in Luria broth-grown H10407. Since adherence may be multifactorial, it was not surprising to find that the effect of *tib* deletion was greater on invasion than on adherence.

The *tib* locus is not homologous to invasion genes from other pathogens. The size of TibA is very similar to that of invasion from *Yersinia pseudotuberculosis* (19 [103 kDa]). However, no homology was observed between *tib* probe I and *inv* plasmid pRI203. Additionally, several monoclonal antibodies that recognize outer membrane localization or cell attachment epitopes of invasin (24) did not recognize TibA in immunoblots.

Invasion DNAs from other enteric pathogens were also analyzed for homology to the *tib* locus. Probe I did not hybridize to pTY19 (*invA*, *-B*, *-C*, and *-D* from *Salmonella typhi*), the invasion plasmid of *Shigella flexneri* (the *ipa* genes), or pCVD437 (*eaeA* gene from enteropathogenic *E. coli*).

As described above, two regions of the *tib* locus appeared to be necessary for invasion: the *tibA* gene and the transposon-identified upstream region. Hybridization probes were generated for each of these regions (probes III and II, respectively [Fig. 7]). *Bam*HI digests of genomic DNAs from a variety of pathogenic and nonpathogenic organisms were examined for homology to these probes. The organisms examined in this study included *Aeromonas hydrophila*, *Bordetella pertussis*, *Campylobacter jejuni*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Plesiomonas shigelloides*, *Proteus mirabilis*, *Salmonella typhi*, *Salmonella typhimurium*, *Shigella flexneri*, *Shigella dysenteriae*, *Shigella boydii*, *Shigella sonnei*, *Staphylococcus aureus*, *Vibrio cholerae*, *Vibrio vulnificus*, *Vibrio parahaemolyticus*, *Vibrio mimicus*, *Vibrio fluvialis*, *Vibrio hollisae*, *Yersinia pestis*, *Yersinia pseudotuberculosis*, *Yersinia enterocolitica*, and the *E. coli* strains listed in Table 3. The only strains that hybridized with the probes were ETEC that expressed CFA/I (Table 3). Response to the probes did not correlate with other known factors such as O or H antigens or toxin production. The size of the *tib*-homologous *Bam*HI fragment was about 5.8 kb and was the same in all hybridizing strains (Fig. 10 and data not shown). The correlation of *tib* with CFA/I appears to be strong but not universal: CFA/I-producing strains from serogroup O153 did not hybridize with the probes, whereas the probes hybridized to CFA/I-producing strains from serogroups O6 and O128 but not to strains from these serogroups if they produced a different colonization factor (Fig. 10).

DISCUSSION

The *tib* locus from ETEC H10407 directs the synthesis of TibA, a 104-kDa OMP. Subcloning, transposon mutagenesis, and complementation experiments show a direct correlation between the production of TibA and the ability of HB101 to invade epithelial cells. These results suggest that TibA is required for the adherence and invasion phenotypes of the *tib* locus. The position of the *tibA* gene within the *tib* locus was identified by T7 induction and complementation experiments. DNA sequences upstream of the *tibA* gene also are required for *tib*-mediated invasion and adherence. This upstream region appears to be involved in the regulation of the *tibA* gene. TibA is not synthesized in the absence of the upstream region; however, providing a copy of this region in *trans* restores TibA production. This result suggests that loss of TibA synthesis upon deletion or mutagenesis of the upstream region is not

TABLE 3. Summary of *tib* hybridization experiments with *E. coli* strains

Strain[a]	Production of CFA (CS)[b]	Serotype	Response to probes II and III[c]	Source[d]
ETEC				
DS 244-1	I	O6:H16	+	WRAIR
M633C1	I	O20:Nm	+	CVD
15758a	I	O78:H10	+	CVD
H10407	I	O78:H11	+	WRAIR
Tx1	I	O78:H12	+	AFRIMS
DS 229-1	I	O128:H12	+	WRAIR
DS 67-1	I	O153:Nm	−	WRAIR
DS 99-5	I	O153:H10	−	WRAIR
H410C1	I	Rough:Nm	+	CVD
M109C2	I	Rough:H12	+	CVD
M424C1	II (1, 3)	O6:H16	−	WRAIR
DS 7-3	II (3)	O8:H9	−	WRAIR
DS 220-4	II (2, 3)	O11:H33	−	WRAIR
DS 373-2	II (2, 3)	O18:Nm	−	WRAIR
DS 207-2	II (1, 3)	O22:Nm	−	WRAIR
DS 300-1	IV (4, 6)	O8:Nm	−	WRAIR
B4106-1	IV (6)	O27:H7	−	AFRIMS
DS 39-5	IV (6)	O115:H35	−	WRAIR
DS 349-1	IV (6)	O128:Nm	−	WRAIR
B7A	IV (6)	O148:H28	−	WRAIR
DS2-1	IV (6)	O159:Nm	−	WRAIR
E17018	IV (5, 6)	O167:H5	−	WRAIR
M415C1	Non-I	O2:Nm	−	CVD
E2539-C1	Non-I	O25:Nm	−	AFRIMS
10614c	Non-I	O78:Nm	−	CVD
Scott	Non-I	O78:K80	−	CVD
EDL 903	Non-I	O88:H25	−	AFRIMS
EPEC				
607-54		O55:H6	−	AFRIMS
B170		O111:Nm	−	AFRIMS
833-90		O119:H6	−	AFRIMS
3336-54		O127:Nm	−	AFRIMS
E2348		O127:H6	−	WRAIR
RDEC-1			−	WRAIR
EIEC (2 strains)			−	AFRIMS
EHEC (7 strains)		O157:H7	−	WRAIR, AFRIMS
EAggEC				
17-2		O3:H2	−	CVD
042		O44:H18	−	CVD
JM221		O93:H33	−	CVD
309-1-1		O130:H27	−	CVD
103-1-1		O148:H28	−	CVD
Normal intestinal isolates of *E. coli* (4 strains)			−	CVD
Other *E. coli*				
HB101			−	WRAIR
DH5α			−	WRAIR
B			−	WRAIR

[a] EIEC, enteroinvasive *E. coli*; EHEC, enterohemorrhagic *E. coli*; EAggEC, enteroaggregative *E. coli*.
[b] Production of CFA and *E. coli* surface (CS) antigen by ETEC strains.
[c] +, hybridization to probes II and III (see Fig. 7); −, no hybridization.
[d] AFRIMS, Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand; CVD, Center for Vaccine Development, Baltimore, Md.; WRAIR, Walter Reed Army Institute of Research.

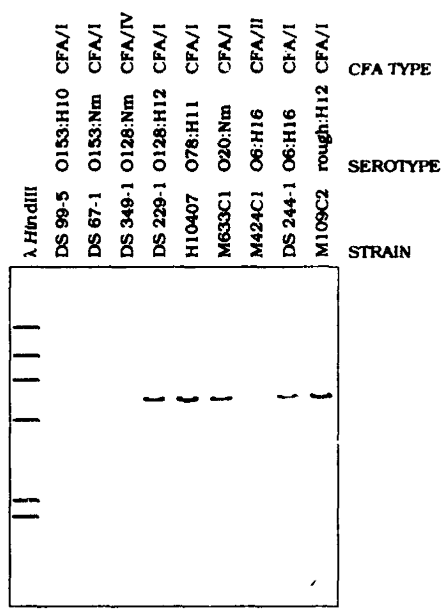

FIG. 10. Hybridization of genomic DNAs with probe II. Genomic DNA from ETEC strains was digested with *Bam*HI, electrophoresed through a 0.6% agarose gel, transferred to nitrocellulose, and then probed with digoxigenin-labeled *tib* probe II (Fig. 7). The positions of λ *Hin*dIII standards are indicated on the left of the gel. Hybridizations with probe III gave identical results.

due to polarity effects on *tibA* transcription but is due to the loss of a regulator that is required for *tibA* transcription.

The *tib* locus sequences upstream of the *tibA* gene also may be required for modification of the TibA protein. In the absence of the upstream region, *tibA* transcription from an exogenous promoter results in the synthesis of a 100-kDa form of the TibA protein that is found in the outer membrane but is incapable of directing epithelial cell invasion. Providing a copy of the upstream region in *cis* or in *trans* restores invasion and production of the 104-kDa form of the TibA protein, regardless of an exogenous promoter.

Subcloning and complementation experiments indicate that the *tib* locus occupies up to 6.7 kb of DNA, 2.9 of which would be necessary for the *tibA* gene. The remaining 3.8 kb could be sufficient to contain two additional *tib* genes coding for a *trans*-acting regulator and a modifying activity. No major proteins other than TibA were observed in the T7 promoter experiments or in SDS-PAGE analyses of whole-cell extracts and membrane fractions. Other *tib* locus proteins may be unstable or may be produced in insufficient quantities to be readily visualized. Experiments to identify other *tib* locus genes are ongoing.

The size and function of the TibA protein indicate that it may be very similar to invasin from *Y. pseudotuberculosis*. Invasin is a 103-kDa OMP that binds to the $\alpha_{3-6}\beta_1$ integrin family of cell adhesion receptors and thereby directs epithelial cell penetration (18, 19). However, monoclonal antibodies against membrane localization and cell attachment epitopes of invasin do not recognize TibA in immunoblots. Additionally, *tib* probe I did not hybridize to the gene encoding invasin nor did probes II and III hybridize to genomic DNA from *Yersinia* species. The *eaeA* gene of enteropathogenic *E. coli* (EPEC) encodes intimin, a 94-kDa membrane protein that is necessary for epithelial cell invasion by EPEC and for the attaching and

effacing lesions that are characteristic of EPEC infections (13, 20, 21). The amino acid sequences of invasin and intimin are highly similar in a region thought to be the membrane localization domain of invasin (42). *tib* probe I did not hybridize to *eaeA* nor did probes II and III hybridize to genomic DNA from EPEC isolates. These results suggest that TibA may be distinct from the invasin family of proteins, but a more detailed genetic and biochemical analysis of TibA is required in order to draw this conclusion.

At the level of DNA homology, the *tib* locus also is distinct from *S. typhi* and *S. flexneri* invasion determinants. The *S. typhi* invasion loci *invA*, *-B*, *-C*, and *-D* span about 30 kb of chromosomal DNA and direct HB101 to penetrate epithelial cells (9). The invasive phenotype of *Shigella* species requires a 37-kb region of a 180- to 210-kb plasmid (27). This region contains the *ipa* genes, which encode several immunogenic membrane-associated proteins that are necessary for invasion (3, 14, 27). *tib* probe I did not hybridize to the *S. typhi inv* loci or the invasion plasmid from *S. flexneri*. Additionally, *tib* probes II and III did not show homology to genomic DNA from *Salmonella* or *Shigella* species.

The *tib* locus may be unique to ETEC strains and may be associated with CFA/I expression. Strains from within the same serogroup producing different CFA types would only hybridize with *tib* probes II and III if they produced CFA/I. However, *tib* does not appear to be diagnostic for CFA/I in that some CFA/I-positive serogroups may not hybridize with the probes (e.g., O153). In the strains that responded to the probes, there was a single *Bam*HI fragment of identical or nearly identical size. Although the hybridizing strains are geographically diverse (originating from locations such as Chile, Thailand, and Saudi Arabia) the similarity in size of the *tib*-homologous fragment indicates that this locus is conserved in strains that contain it. While the CFA/I genes are plasmid encoded (11, 32), the *tib* locus in H10407 is chromosomally encoded (10). Since the hybridization experiments reported here were performed with genomic DNA preparations, it is uncertain if the *tib* locus in all hybridizing strains is chromosomally encoded. The nature of the association between the *tib* locus and plasmid-encoded genes is unknown.

Expression of the *tib* locus may be regulated in H10407. Three lines of evidence support this hypothesis. (i) Whole-cell extracts and membrane preparations indicate that TibA is poorly or not synthesized by H10407 grown under routine laboratory conditions. (ii) Membranes purified from wild-type and Δ*tib1* deletion strains appear to be identical. (iii) *tib*-positive HB101 can invade epithelial cell lines not penetrated by H10407. The direct correlation between epithelial cell invasion and TibA expression suggests that if the *tib* locus genes are expressed by a strain, that strain would invade receptive epithelial cells. If the correlation between TibA production and epithelial cell invasion can be extended to H10407, it would suggest that the *tib* locus genes are not expressed by laboratory-grown H10407. Exposure of H10407 to invasion-permissive epithelial cells could result in the synthesis of *tib*-associated proteins. The *tib* locus appears to be deregulated in HB101, allowing for visualization of TibA and for invasion of nonpermissive epithelial cells.

The *tib* locus is one of two loci in H10407 that direct epithelial cell penetration (*tia* and *tib*). Deletion of the *tib* locus from H10407 reduced invasion to 15% of the wild-type level, showing that this invasion system plays an important part in epithelial cell entry by this strain. Hybridization experiments indicate that the *tib* invasion system is not present in some ETEC strains, including strains that previously have been shown to be invasive (10). Epithelial cell penetration by these strains may be due to the activity of the *tia* invasion system or some other undescribed mechanism.

The role, if any, of epithelial cell invasion in ETEC pathogenesis has yet to be determined. Although there is no histopathological evidence for invasion, it has been shown that nontoxigenic colonizing ETEC strains can cause diarrhea (26, 28, 29, 33, 40). The route through which this effect is elicited is unknown. Epithelial cell penetration may play a role in enterotoxigenic disease by allowing the organisms to reach an intracellular environment in which they might induce unidentified toxins or other virulence factors. Additionally, intracellular organisms might more effectively deliver known toxins. Finally, epithelial cell invasion might play a role in prolonged diarrheal illness by providing a niche for survival. H10407 strains bearing deletions of both the *tia* and *tib* invasion loci are being constructed in order to determine the relevance of epithelial cell invasion for ETEC pathogenesis.

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